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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Pharmaceutical Containing the p40 Subunit of Interleukin 12

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Abstract of the disclosure

Pharmaceutical containing the p40 subunit of interleukin 12

The present invention relates to a pharmaceutical containing the p40 subunit of interleukin 12. This pharmaceutical is particularly suitable for use in the treatment of disorders which are associated with dysregulation of the immune-system.

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The present invention relates to a pharmaceutical containing the p40 subunit of interleukin 12. This pharmaceutical is particularly suitable for use in the treatment of disorders which are associated with dysregulation of the immune system.

Immunereactions can be supported by different T cell populations. Depending on the type of the immunereaction, T-cell help is preferentially provided either by Type 1 T-helper cells (TH1) or by Type 2 T-helper cells (TH2). As far as is known to date, TH1 cells differ, in particular, from TH2 cells by producing different cytokines.

Dunn et al. (J. Immunol. 142:3847 ff, 1989) were able to demonstrate as early as 1989 that TH1 clones produce gamma-IFN, provided they were cultivated in the presence of accessory cells and IL-2. In addition, Germann et al. (Eur. J. Immunol., 8:1857-1862, 1991) were able to demonstrate that a soluble mediator is required for the synthesis of gamma-IFN by TH1 cells. It emerged from comparative investigations that the mediator designated by Germann et al. as TSF is identical to murine IL-12. Recently, Mengel et al. (Eur. J. Immunol. 22:3173-3178, 1992) were then likewise able to describe, in the supernatant of the activated murine B cell lymphoma line A-20, a soluble factor which stimulates gamma-IFN production in T cells. The soluble factor described by Mengel in the A-20 supernatant is compared functionally by Wolf, S.F. et al., (J. Immunol., 156:3074, 1991) with a so-called "human natural killer cell stimulatory factor" (NKSF)" and postulated to be the murine analog of human IL-12. It is known that both IL-12 and NKSF, which are potentially identical, stimulate gamma-IFN synthesis. The function of IL-12 (corresponding to NKSF) is

naturally not limit d to the stimulation of gamma-IFN production. Inter alia, IL-12 (corresponding to NKSF) also affects the function of natural killer cells (so-called NK or LGL cells) and IgE production, and increases IL-12-induced proliferation of resting peripheral mononuclear cells.

It is also known that IL-12 is composed of two different subunits which are designated p35 and p40 (Podlaski, F. J. et al. (1991) Arch. Biochem. Biophys. Vol. 294, No. 1, pp. 230-237). Murine IL-12 was also found to have an almost identical structure (Schoenhaut D. S. et al. (1992) J. Immunol. Vol. 148, No. 11, pp. 3433-3440). It has been demonstrated that while the p40 subunit of IL-12 does not possess any IL-12-specific bioactivity on its own, the p40 subunit is of considerable importance for the bioactivity of the complete IL-12 molecule. It has been speculated that the p40 subunit interacts directly with the cellular IL-12 receptor.

According to a whole series of publications, dysregulation of cytokine-production and cytokine-effects may cause acute and chronic disorders of the immune-system and their sequelae. The influencing of cytokine-mediated activities could therefore represent a starting point for a therapeutic approach.

The present invention is therefore based on the object of making available a pharmaceutical which can advantageously be employed for treating pathological conditions which are associated with dysregulation of IL-12-mediated activities.

It has now been found, surprisingly, that the p40 subunit of IL-12 can be employed for inhibiting IL-12-mediated activities. Because of this, it is now possible to achieve therapeutic effects by intervening in the said regulatory mechanisms.

The present invention thus relates to a pharmaceutical containing a natural or recombinant, possibly modified, p40 subunit of mammalian IL-12. Within the scope of the invention, modified p40 subunits are active parts or variants of p40, it being possible to demonstrate activity in an animal model (mouse) or in vitro, for example, in accordance with the processes given in the examples.

A preferred embodiment of the invention relates to a pharmaceutical containing a p40 subunit of human origin.

A further aspect of the invention relates to a diagnostic agent containing a natural or recombinant p40 subunit of mammalian IL-12, preferably from tissues or cells of human origin.

The present invention furthermore relates to the use of the natural or recombinant, possibly modified, p40 subunit of mammalian IL-12, preferably of human origin, for preparing a pharmaceutical for use in the treatment of pathological conditions which are associated with dysregulation of IL-12-mediated activities, such as, for example, autoimmune diseases, such as systemic lupus erythematodes, Wegener's syndrome or rheumatic arthritis, bacterial or viral infections, and certain solid tumors or leukemias.

A further aspect of the invention relates to the use of the natural or recombinant, possibly modified, mammalian IL-12 p40 subunit in a process for detecting IL-12 in body fluids, for example from humans. This process is based on a specific inhibition of IL-12 activity by p40 and can be effected, for example in an analogous manner to Examples 1 and 2, such that the IL-12 which is employed derives from the sample to be analyzed and p40 is added in a defined quantity.

All the said processes are preferred which make use of natural or recombinant p40 of human origin.

A further aspect of the invention relates to a diagnostic agent containing IL-12 for detecting the IL-12 p40 subunit. This diagnostic agent can be used, for example in an analogous manner to Examples 1 and 2, such that the p40 inhibitor activity which is employed derives from the sample to be analyzed and IL-12 is added in a defined quantity.

In addition, the invention is elucidated by the examples and the claims.

Isolation of IL-12 and p40:

For the isolation of recombinant murine IL-12 and the p40 subunit of mIL-12, mRNA was isolated using standard techniques (Sambrook, J., Fritsch, E., Maniatis, T., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) from murine spleen cells and then converted into double-stranded cDNA. Using the primers described in Schoenhaut et al. (Schoenhaut, D., Chua, A.O., Wolitzky, A.G., Quinn, P.M., Dwyer, C.M., McComas, W., Familletti, P.C., Gately, M.K, Gubler, U. (1992). J. Immunol. 148, 3433), and employing the experimental conditions given in this publication, a PCR was carried out which resulted in the generation of an approximately 600 base-pair fragment.

The PCR fragment was sequenced by standard techniques (Sambrook J., Fritsch, E., Maniatis, T., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The result of this was that the experimentally determined cDNA sequence was found to be identical to the published sequence for the murine p40 subunit. The PCR fragment for the p35 subunit was isolated and sequenced in an analogous manner. In order to confirm that the biological activities described below do indeed result from IL-12 and/or the p40 subunit, the PCR fragments were cloned, individually or in combination, into the vector pABstop and stably expressed in BHK-21 cells using a standard process (Zettlmeißl G., Wirth, M., Hauser, G., Küpper, H.A. (1988) Behring Inst. Mitteilungen (Communications) 82, 26).

It was possible to isolate both biologically active mIL-12 and a biologically active p40 subunit of mIL-12 from culture supernatants of transfected BHK-21 cells.

Supernatant of the murine B-cell lymphoma line A-20 (American Type Culture Collection, ATCC TIB208), which was activated in accordance with the method described by Mengel et al. (see above), served as the source of natural IL-12. The soluble mediator described by Mengel in the A-20 supernatant is compared functionally with NKSF and is regarded as the murine analog of

human IL-12. Spleen cell preparations which were prepared and activated in accordance with the method described by Germann et al. (see above) represented a further source of natural IL-12. Comparative investigations demonstrated that the TSF described by Germann et al. is identical to mIL-12.

It was possible to isolate a hybridoma cell which secreted the p40 subunit of murine IL-12 and this was used as the natural source of this subunit. To prepare this hybridoma cell, 1-10 x 10^6 murine T cells, which had been cultivated in the presence of syngeneic monocytes (1 x 10^5/ml) and recombinant murine GM-CSF (50 ng/ml), were injected subcutaneously together with CFA (complete Freund's adjuvant) into female rats (Lewis strain, Zentralinstitut für Versuchstierkunde (Central Institute for Experimental Zoology), Hannover). Two further immunizations took place at intervals of 2 weeks on each occasion, when the same cell quantities were injected intraperitoneally. The animals were sacrificed 3 days after the last injection and the spleen cells were fused with the cells of the murine myeloma cell line SP2/FO in accordance with the well known standard procedure of Köhler and Milstein (Nature 256, p. 495ff; 1975). The growing hybridoma cell was selected in accordance with standard procedures. Investigations which were carried out in analogy with the experiment presented in Example 1 demonstrated that supernatants of one of the isolated hybridoma were able to inhibit gamma-interferon release. Following the published process of Kobayashi et al. (Kobayashi, M.; Fritz, L., Ryan, M., Hewick, R.M., Clar, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B., Trinchieri, G. (1989) J. Exp. Med. 170, 827), a secreted protein was purified from the culture supernatant of this hybridoma cell. After purification by reverse-phase HPLC, the isolated protein fraction was fractionated by SDS-PAGE, a dominant protein band being found in the region of about 40-45 kD. Sequence comparisons confirmed that this isolated protein is identical to the p40 subunit of murine IL-12.

The pharmaceutical is finally prepared by a process which is known per se to the person skilled in the art. The IL-12 p40 subunit (= the active compound) is employed in an effective concentration either as such or in combination with suitable pharmaceutical additives or auxiliary substances as well as physiologically acceptable solvents.

Example 1:

Inhibition by p40/IL-12 of the IL-12-induced release of gamma-IFN

In order to induce the release of gamma-IFN, 5 x10^6 spleen cells from BALB/c mice were cultivated for 48 h. in the presence or absence of recombinant or natural mIL-12 and IL-2 in concentrations given. After 48 h. of culture, the supernatant was harvested from the spleen cells and centrifuged until it was cell-free. The content
of gamma-IFN in the supernatant was determined in commercially available ELISA systems (e.g. IntertestTM-gamma, Mouse IFN-gamma ELISA kit; Genzyme). The gamma-IFN from the culture supernatant of activated spleen cells was quantified by comparison with recombinant murine gamma-IFN (Genzyme). A typical experiment is presented in Figure 1. As the data demonstrate, cultivation of murine spleen cells with IL-12 led, in a dose-dependent manner, to the release of >5 ng/ml of gamma-interferon. However, if the spleen cells were preincubated, at the beginning of the culture, with recombinant murine p40/IL-12 or with hybridoma supernatant which contained the natural p40 subunit of mIL-12, and then stimulated with mIL-12, the IL-12-dependent synthesis of gamma-IFN was inhibited by at least 50 %. This example, presented here, demonstrates that both recombinant p40/IL-12 and hybridoma supernatant containing the natural p40 subunit of mIL-12 are able to inhibit the IL-12-induced synthesis of gamma-INF.

Example 2:

Inhibition by p40/IL-12 of the IL-12-induced activity of NK cells

Spleen cells from C57BL6 mice were cultivated in serum-free Iscove's medium at a cell density of 5-10x10^6 cells/ml in 24-well Costar plates at 37°C for 18 h. The spleen cells were cultivated in different concentrations of recombinant or natural murine IL-12. After 18 h., the cells were harvested, the number of living cells was determined by Trypan Blue staining, and the cytolytic activity was determined in a 5-hour ^{51}Cr release test. YAC-1 cells (ATCC TIB160) were

used as target cells. 51Cr labelling of target cells, and the ^{51}Cr-releas assay were carried out according to standard methods (e.g. Schoenhaut D.S. et al. (1992) J. Immunol. Vol. 148, No. 11, pp. 3433-3440). The ratios of effector cells to target cells were typically 100:1, 50:1, 25:1 and 12.5:1. The % specific cytolysis was calculated as (% lysis of the experimental group - % spontaneous lysis): (% maximum lysis - % spontaneous lysis) x 100. By preincubating C57BL/6 spleen cells with IL-12 it was possible to increase the specific cytolysis by at least 5-fold as compared with the starting control (ratio 50:1) . However, if, under the same culture conditions, the spleen cells were additionally preincubated with recombinant or natural murine p40/IL-12, the IL-12-dependent cytolysis was then inhibited by at least 50 %.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A pharmaceutical containing a natural or recombinant, possibly modified, p40 subunit of mammalian IL-12.

2. A pharmaceutical as claimed in claim 1, containing p40 of human origin.

3. A diagnostic agent containing a natural or recombinant, possibly modified, p40 subunit of mammalian IL-12.

4. A diagnostic agent as claimed in claim 3, containing p40 of human origin.

5. Use of the natural or recombinant, possibly modified, p40 subunit of mammalian IL-12 for preparing a pharmaceutical for the treatment of pathological conditions which are associated with dysregulation of IL-12-mediated activities.

6. The process as claimed in claim 5, wherein p40 of human origin is used.

7. Use of the natural or recombinant, possibly modified, mammalian IL-12 p40 subunit in a process for detecting IL-12 in human body fluids.

8. The process as claimed in claim 7, wherein p40 of human origin is sed.

9. A process for detecting the IL-12 p40 subunit in body fluids.

10. A process for preparing a pharmaceutical as claimed in claim 1,

wherein the active compound is brought, together with a physiologically acceptable solvent and, where appropriate, further additives or auxiliary substances, into a form which is suitable for arenteral administration.

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Figure 1